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SHORT SEQUENCE RNAS AND LIPID CONJUGATES AS NOVEL DRUG DELIVERY SYSTEMS

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ABSTRACT

Lipids are an integral part of the cell membrane and synthetic lipids have become increasingly important as formulation excipients and as active ingredients *per se*. The present article summarizes particular features of commonly used phospholipids and their application spectrum and elucidates current strategies to improve bioavailability and disposition of administered microRNA or short interfering RNA (miRNA)/siRNA). Technological strategies to achieve these effects are highly diverse and offer various possibilities of liquid, semi-liquid and solid lipid based formulations for novel nucleotide based delivery optimization. The complexity of microRNA-mediated pathway control has burgeoned since the discovery that miRNAs are found in the extracellular space and constitute a form of cell to cell communication. miRNAs have been found in plasma, urine, and saliva and have recently been shown to be carried on lipoproteins. Although our understanding of the cellular machinery responsible for the secretion of miRNA is still incomplete, there is little doubt that extracellular miRNAs delivered as conjugates with lipids will hold tremendous potential as both diagnostic and therapeutic agents.

KEYWORDS: Nanoparticles, phospholipids, microRNA, drug, membrane.

INTRODUCTION

It is well understand that the secretion of miRNAs is a controlled, active and specific process. miRNAs can be packaged into lipid-based carriers such as exosomes, microparticles, or apoptotic bodies and have been found on lipoproteins like the high- and low-density lipoprotein for e.g HDL and LDL, respectively.[1,2] miRNAs are a specific class of noncoding RNA (ncRNA) and are defined as small, 20–22 nucleotide RNA molecules that are processed from a much larger primary transcript. Once processed into their mature form, miRNAs generally bind to complimentary sequences in the 3′ untranslated region (UTR) of specific genes but can also bind to other regions of the gene including the 5′ UTR and the coding region via mRNA destabilization and/or protein translation inhibition, miRNAs mediate silencing of their bound targets. Recently, the importance of miRNAs in the extracellular space has been exemplified by a number of studies showing specific and regulated export of miRNA from the cell, and the uptake and functional consequences in recipient cells. However, miRNAs that remained associated with their lipid carrier and/or protein complexes were indeed highly resistant to degradation.

Novel drug delivery systems are being researched for optimal bioavailability and lesser side effects with targeted therapeutic efficacy. In this regard, the lipid nanoparticles loaded with microRNAs–phospholipid complex are being tried out to evaluate the action in the tumor site for e.g. hepatoma and to exploit a feasible anti-tumor delivery system for practical purpose.[3] Recent experiments suggest that formulations not only show satisfactory encapsulation efficiency and high drug loading capacity, but also the components of lipid nanoparticles were biodegradable and physiological lipids, which depicted low toxicity and low cytotoxicity, such as lecithin and injectable grade soyabean oil. And these materials are clinically available for several decades; thus these formulations show substantial potential for therapeutic application. Phospholipids for e.g phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS) have a special amphiphilic character. When placed in water, they form various structures depending on their specific properties. Mostly, they form micelles or are organized as lipid bilayers with the hydrophobic tails lined up against one another and the hydrophilic head-group facing the water on both sides The possibility that short sequence RNAs can be conjugated with lipid products is a challenging but practicable one. This review examines the possible role of lipid nanoparticles loaded with miRNA-phospholipid conjugates in diagnostics and therapeutics.[4]

RNAs and lipid based carriers

The evidence that miRNA originate from the cell surface, microvesicles contain very similar lipid content as the plasma membrane of the parental cell type, most often enriched with phosphatidylserine (PS) and PC. Similarly, the protein content of microvesicles is highly related to the originating cell and there may be less selective cargo loading in these vesicles compared with exosomes.[5] Nevertheless, microvesicles do carry functional protein, mRNA and miRNA cargo, and delivery of these components to neighboring cells has significant effects on recipient cell function. The invention is based, in part, on the discovery that siRNA can be reversibly conjugated to a phospholipid, such as a phospholipid within a liposome or a micelle and the siRNA can be unconjugated upon exposure to reducing conditions, such as inside a cell.

Accordingly, in one aspect, the disclosure features a conjugated siRNA composition comprising a micelle or a liposome, the micelle or the liposome comprising phospholipids and an siRNA reversibly conjugated to a first phospholipid of the micelle or the liposome.

In some delivery systems, the siRNA is reversibly conjugated to the first phospholipid by a disulfide bond. In certain embodiments, the siRNA is unconjugated from the first phospholipid upon exposure to reducing conditions. Apoptotic bodies are another variety of lipid-encapsulated vesicles known to carry miRNA. These types of vesicles are generally much larger (~500 nm to >2000 nm) and have a heterogeneous size distribution. As the name suggests, apoptotic bodies are released at the early stages of apoptosis and contain both a lipid bilayer derived from the plasma membrane and cytoplasmic contents that originate from the parent cell. Similar to microvesicles, apoptotic bodies contain PS on their cell surface, which signals to phagocytic cells like macrophages to engulf and clear the cellular debris.[6] Although it was suggested many years ago that apoptotic bodies contain nucleic acids.

In certain embodiments, the siRNA reversibly conjugated to the first phospholipid has increased stability relative to the same siRNA not conjugated to the first phospholipid.

Lipid vesicles as miRNA carriers

Any suitable vesicle-forming lipid e.g., naturally occurring lipids and synthetic lipids can be utilized in the liposomes and micelles. Suitable lipids include, without limitation, phospholipids such as phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidyethanolamine (PE), phosphatidylserine (PS) and phosphoethanolamine; sterols such as cholesterol; glycolipids; sphingolipids such as sphingosine, ceramides, sphingomyelin, and glycosphingolipids (such as cerebrosides and gangliosides). The miRNA can be specifically modified such that it can be conjugated to that phospholipid. For example, one or more nucleotides of an siRNA can also be modified to include a carboxylic acid functionality, and a phospholipid can be modified with an amine moiety. The amine would then be capable of reacting with the carboxylic acid functionality on the miRNA, using procedures known in the art, to form an amide linkage between the phospholipid and miRNA.[7]

Lipid nanoparticles (LNP) for siRNA

The usage of cationic lipids bearing a positive charge to mediate the intracellular delivery of nucleic acid was initially described by Felgner & Ringold. However the complexes proved of limited use only in vivo systems as higher size and surface charge resulted in rapid clearance and toxic side effects. A newer advancement was the development of ionizable cationic lipids with apparent pKa in the value of 7 or lower. alongwith the development of the techniques to encapsulate nucleic acid polymers into stable LNP with diameters of 100nm or even lesser. Thus ionizable lipids displayed neutral surface charge and much lesser toxicity.[8-10] Ionizable cationic lipids for e.g DODAP contribute in stimulating intracellular release from endosomes. Alternatively, the siRNA can be modified with an amine and reacted with a carboxylic functionality on a phospholipid to form an amide linkage. Other examples of other functionalities that react with amines are acyl chlorides, acid anhydrides, esters and carboxylic salts.[11,12]

The siRNA-phospholipid conjugate can be linked, for example, via amide linkages. One particular mechanism of producing carbamide linkages includes the use of alkyl chloroformate groups. In one such non limiting example, a phospholipid with a chloroformate group is reacted with an siRNA having an amine group functionality in the presence of a base. The resulting compound is a siRNA-phospholipid conjugated by a carbamide linkage.

The siRNA can also be chemically conjugated to a phospholipid via an ester bond. A common method of performing such esterifications is the use of Steglich esterification. In this example, siRNAs are modified with carboxylic acid functionalities at one or more positions. The carboxylic acids are then activated with dicyclohexylcarbodiimide. Subsequently, 4-dimethylaminopyridine is used to catalyze an acyl-transfer with a hydroxyl group on the phospholipid.

In other instances, a liposome or micelle reversibly conjugated to an miRNA, described herein, is formulated for intravenous administration.[13,14] Compositions for intravenous administration can comprise a sterile isotonic aqueous buffer. The compositions can also include a solubilizing agent. Compositions for intravenous administration can be similar to local anesthetic such as lignocaine to lessen pain or induce localized anesthesia at the site of the injection. The ingredients can be supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a

hermetically sealed container such as an ampule or sachette indicating the quantity of active agent. Where a liposome or micelle described here in is administered by infusion, it can be dispensed, for example, with an infusion bottle containing sterile pharmaceutical grade water or saline. Where a liposome or micelle described herein is administered by injection, an ampule of sterile water for injection or saline can be provided so that the ingredients can be mixed prior to administration.

Preparation of miRNA encapsulated liposomal nanoparticles

Liposomes can be prepared by using combination of DOTAP, cholesterol and DSPE-PEG 2000-cyanur. Briefly, the lipids i.e DOTAP, cholesterol and DSPE-PEG-2000-cyanur (1:1:0.1 molar ratio) are dissolved in ethylalcohol (40–100 μg/μL) and are dispersed into aqueous solution of molecular grade containing let-7a miRNA as described earlier.[15] The weight ratio of the miR to liposomal nanoparticles (LNP) is kept at 1:09. The LNP and miRNA are well dispersed and vortexed for the formation of stable miRNA–LNP complex. The encapsulation of miRNA into the LNP is determined by agarose gel electrophoresis. Free miRNA, miRNA–LNP and miRNA–ephrin-A1–LNP complex are electrophoresed on a 1.1% agarose gel and bands arevisualized using Chemidoc-MP System (Bio-Rad Laboratories, USA).

Clinical trials of lipid-miRNA conjugates

Liposomes are biodegradable and could be used to deliver high concentrations of the gene dosage to the tumor tissue. Liposomes composed of the cationic lipid DOTAP (N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl-sulfate) have been shown to be the effective carrier for the anionic nucleotides RNA and DNA. Due to the overall cationic electrostatic charge on the lipid bilayers, cationic liposomes provide advantages that include high encapsulation efficiency of nucleotides and high cellular uptake. Cationic liposomes have been demonstrated to selectively accumulate in angiogenic endothelial cells in tumors and to be internalized by endocytosis after intravenous injection.[16] This gene delivery system is now being investigated in a Phase I clinical trial. Synthetic lipid nanoparticles have also been used for miRNA delivery to cells and tissues. The lipid bilayer of the nanoparticle can easily penetrate the plasma membrane into the recipient cell and thus achieves efficient cellular uptake. Bader *et al*. delivered miR-34a to lung tumors in mice using liposomes and demonstrated 60% decrease in tumor area after delivery.[17] The therapeutic effect of miR-34a delivery is now being studied in a Phase I clinical trial using an amphoteric liposome. Pre-clinical work by Mirna Therapeutics has demonstrated potent anti-tumor effects by introducing miR-34a mimics into a variety of mice cancer models. The usage of miRNA mimics for systemic delivery is challenging compared to anti-miRNA drugs.[18] miRNA mimics need to be double-stranded in order to be processed correctly by the cellular RNAi-machinery and therefore cannot be administered "naked". Successful delivery therefore requires complex delivery vehicles mimicking physiological settings where miRNAs reside in microvesicles or exosomes. For MRX34, Mirna therapeutics has developed custom nanoparticle liposomes.[19,20] According to company information (MIRNA THERAPEUTICS) these liposomes increase stability, enhance delivery and prevent immune response effects. Extensive pre-clinical testing of MRX34 in mouse models of hepatocellular carcinoma using liposomes has provided promising outcomes and the upcoming clinical trial is recruiting patients with non resectable primary liver cancer or metastatic cancer with liver involvement.

CONCLUSIONS

MicroRNAs have come a long way since the initial discoveries two decades ago. 20–30 nucleotide RNA molecules have emerged as critical regulators in the expression and function of eukaryotic genomes. Two primary categories of these small RNAs—short interfering RNAs (siRNAs) and microRNAs (miRNAs)—act in both somatic and germline lineages in a broad range of eukaryotic species to regulate endogenous genes and to defend the genome from aberrant nucleic acid proteins. Recent advances have revealed unexpected diversity in their biogenesis pathways and the regulatory mechanisms that they access. Our understanding of siRNA- and miRNA-based regulation has direct implications for fundamental biology as well as disease etiology and treatment.Their emerging potential as biomarkers in clinical diagnostics as well as modulators for the treatment of a variety of diseases is truly exciting. In the near future it will become evident as to whether they have the strength to become established as a new molecular diagnostic and therapeutic tools.

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